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Roles of reactive oxygen species in interactions between plants and pathogens

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Abstract The production of reactive oxygen species (ROS) by the consumption of molecular oxygen during host–pathogen interactions is termed the oxidative burst. The most important ROS are singlet oxygen ($^1O_2$), the hydroxyperoxyl radical ($HO_2\cdot$), the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH^-) and the closely related reactive nitrogen species, nitric oxide (NO). These ROS are highly reactive, and therefore toxic, and participate in several important processes related to defence and infection. Furthermore, ROS also play important roles in plant biology both as toxic by-products of aerobic metabolism and as key regulators of growth, development and defence pathways. In this review, we will assess the different roles of ROS in host–pathogen interactions with special emphasis on fungal and Oomycete pathogens.

Keywords Antimicrobial · Cell wall cross-linking · Hypersensitive response · Signal transduction · Gene expression · Successful pathogenesis · Hydrogen peroxide

Abbreviations

ROS	reactive oxygen species
SA	salicylic acid
ET	ethylene
MAPK	mitogen-activated protein kinase
SOD	superoxide dismutase
CWA	cell wall appositions
NO	nitric oxide
JA	jasmonic acid
HR	hypersensitive response
PCD	programmed cell death

Introduction

Generation of ROS, especially hydrogen peroxide (H_2O_2), has been recorded in interactions with a variety of pathogens (Mellersh et al. 2002; Shetty et al. 2003; Thordal-Christensen et al. 1997; Unger et al. 2005). Avirulent pathogens often induce a biphasic ROS accumulation with a small, transient first phase, followed by a continuous phase of much higher intensity that correlates with disease resistance (Lamb and Dixon 1997; Torres et al. 2006). However, three phases of ROS accumulation have been observed in

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some cases, e.g., for *Blumeria graminis* f. sp. *hordei* infecting barley (Hückelhoven and Kogel 2003) and *Septoria tritici* infecting wheat (Shetty et al. 2003). These differences can be attributed to the more complicated development of these fungal pathogens and the influence of the host genotype, which presumably determine whether two or three phases of ROS accumulation occur. Virulent pathogens that avoid or suppress host recognition induce only the transient, first phase of this response (Bolwell et al. 2002). Elicitors of defence responses, often now referred to as microbe or pathogen-associated molecular patterns (PAMPs), also trigger an oxidative burst (Chisholm et al. 2006). There are several potential sources of ROS in plants and different sources of ROS may be activated within a species in different situations depending on the type of stress (Bolwell et al. 2002; Lamb and Dixon 1997). A variety of enzyme systems have been implicated in ROS generation following pathogen recognition, i.e., reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Bedard et al. 2007; Carter et al. 2007; Grant et al. 2000), SOD (Auh and Murphy 1995; Deepak et al. 2006), oxalate oxidases (Hu et al. 2003; Zimmermann et al. 2006), peroxidases (Bindschedler et al. 2006; Bolwell et al. 2002), lipoxygenases (Babitha et al. 2004) and amine oxidases (Allan and Fluhr 1997; Cona et al. 2006; Walters 2003). Stress on of ROS-producing organelles during pathogenesis may also contribute to ROS production during host–pathogen interactions. Mitochondria are normally considered relatively unimportant ROS generators in photosynthesising tissue (Apel and Hirt 2004; Kuźniak and Skłodowska 2005). However, a recent review by Amirsadeghi et al. (2007) discusses evidence that mitochondria are a potential source of ROS in response to biotic stress. Chloroplasts (Kariola et al. 2005) and peroxisomes (Kuźniak and Skłodowska 2005) have also been shown to be important. In this review, we present recent knowledge on the roles of ROS during host–pathogen interactions with special emphasis on fungal and Oomycete pathogens.

Roles of ROS in host–pathogen interactions

ROS have been implicated in many different processes related to pathogen interactions with their hosts. In the initial phases of the interactions, this essentially means involvement in defence processes, whereas at the later stages, during pathogen colonisation, the role of ROS may be more ambiguous.

ROS as antimicrobial agents

ROS, especially H_2O_2, was suggested as an antimicrobial agent during the plant defence response (Apostol et al. 1989; Custers et al. 2004; Legendre et al. 1993; Walters 2003). However, the actual toxicity of ROS in a given plant–pathogen interaction will depend on the sensitivity of the pathogen to the concentration of ROS present (Levine et al. 1994). The amount of extracellular H_2O_2 produced depends on several factors including the nature of the elicitor, the plant species, and age or developmental stages of the plant cells (Legendre et al. 1993; Małolepsza 2005; Nurnberger et al. 1994). Micromolar concentrations of H_2O_2 inhibited spore germination of a number of fungal pathogens *in vitro* (Peng and Kuc 1992). Thus, a concentration of 0.1 mM H_2O_2 completely inhibited the growth of cultured bacteria *Pectobacterium carotovorum* subsp. *carotovorum* (formerly *Erwinia carotovora* pv. *carotovora*), and resulted in >95% inhibition of *Phytophthora infestans* growth (Wu et al. 1995). Shetty et al. (2007) demonstrated by in vitro experiments that 5 mM H_2O_2 inhibited the development of inoculum from 4 day-old *S. tritici* cultures whereas a concentration of about 50 mM was required to inhibit inoculum from 16 day-old cultures. This reflects the ability of the pathogen to tolerate H_2O_2 during the different stages of its life-cycle. Shetty et al. (2007) also demonstrated that in the wheat–*S. tritici* interaction, infiltration of 4 mM H_2O_2 into a susceptible cultivar made it more resistant, symptoms appearing 6 days later than in control plants, whereas infiltration of catalase resulted in symptoms appearing 4 days earlier. It is currently not known whether the effect of H_2O_2 was direct, i.e., by toxicity of ROS, or indirect by affecting signal transduction or defence gene expression.

It is difficult to determine which H_2O_2 concentrations actually inhibit pathogens in planta since the necessary manipulation of the host tissue may itself trigger the production of ROS and/or antioxidants. However, ROS are also toxic to the plant. Thus, soybean suspension-cultured cells remained viable with up to 4 mM H_2O_2, whereas slightly higher levels (6–8 mM) resulted in extensive cell death (Levine et

al. 1994). Therefore, ROS accumulation is tightly regulated by the plant to avoid high concentrations, which could damage the plant tissue (Torres et al. 2006).

Involvement of ROS in signal transduction and gene expression

ROS are involved in different signalling pathways for defence mechanisms, such as triggering of the HR, accumulation of phytoalexins and a number of other defence-response genes (cf. Fig. 1). It has been suggested that ROS are sensed by plants via three mechanisms (Mittler et al. 2004): unidentified receptor proteins; redox-sensitive transcription factors such as natriuretic peptide receptor 1 (NPR1) or heat-shock transcription factors (HSFs); and direct inhibition of phosphatase (Apel and Hirt 2004; Mittler et al. 2004; Neill et al. 2002). ROS signalling is the subject of intense studies, but the role of ROS in signalling is poorly understood. Here, we will give an overview of the initial events involved in signalling in plant–pathogen interactions.

Protein phosphorylation, changes in ion fluxes and the oxidative burst, leading to either HR or defence gene expression, or both, are important events taking place after pathogen infection (Chandra et al. 1996; Jabs et al. 1997; Lamb and Dixon 1997; Sasabe et al. 2000). The earliest reactions of plant cells include changes in plasma membrane permeability, which leads to Ca^{2+} and proton influx and K^+ and Cl^- efflux (McDowell and Dangl 2000). Ion fluxes subsequently induce extracellular production of ROS catalysed by enzymes that act as secondary messengers for the HR and defence gene expression (Lamb and Dixon 1997). Calcium has been shown to be important in signalling. Heteromeric guanosine triphosphate (GTP)-binding proteins and protein phosphorylation/dephosphorylation events are probably involved in transferring the signals from the receptor to calcium channels that activate downstream processes (Legendre et al. 1992). Furthermore, elevation of the cytosolic calcium concentration has been shown to occur during most biotic and abiotic stresses (Price et al. 1996). For example, oxidative stress increased the cytosolic calcium concentration in tobacco (Price et al. 1994) and H_2O_2 induced calcium influx-mediated stomatal closure in *Commelina communis* and *Arabidopsis thaliana* (McAinsh et al. 1996; Pei et al. 2000). In further support of the involvement of calcium in signalling, lanthanide ions (calcium channel blocker) inhibited bacterial elicitor-induced ROS production in tobacco (Baker et al. 1993). Moreover, Urquhart et al. (2007) showed that transient expression of the chimeric cyclic nucleotide-gated ion channel gene ATCNGC11/12 in *Nicotiana benthamiana* gave rise to cell death with characteristics of the HR. Furthermore, it was shown that this gene could function as a Ca^{2+}-conducting channel and that calcium ions were important for the observed cell death. Recently, Ashtamker et al. (2007) showed that nuclei isolated from tobacco were capable of producing H_2O_2. This was dependent on calcium, suggesting that nuclei can be a source of ROS production.

Different models for the action of calcium in the regulation of ROS have been proposed. One model

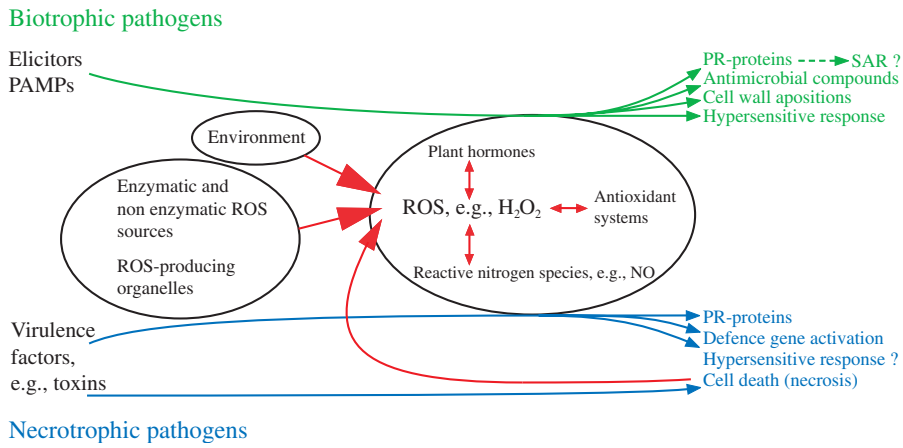

Fig. 1 Putative sources and functions of reactive oxygen species (ROS) in host–pathogen interactions of biotrophic and necrotrophic organisms

suggests that an elicitor interacts with a receptor coupled with a G-protein, which leads to Ca^{2+} influx that activates a Ca^{2+}-dependent protein kinase and ultimately NADPH oxidase (Blumwald et al. 1998). Another model, based on studies of innate immunity in *Arabidopsis*, suggests that pathogens or PAMPs are recognised by (unknown) receptors which trigger an ion (calcium) channel, leading to increases in cytosolic Ca^{2+} and subsequent NO generation (Ali et al. 2007). NO generation, together with other required factors such as an avirulent pathogen and an oxidative burst, could lead to the HR and potentially, diffusion of NO to neighbouring cells could act as a signal that thereby activates further calcium channels.

Activation of the oxidative burst is governed by phosphorylation/dephosphorylation (Lamb and Dixon 1997). Thus, a protein phosphorylation cascade that has been shown to be activated by H_2O_2 is a MAPK cascade, which has an important role in signal transduction (Zwerger and Hirt 2001). H_2O_2 has been shown to activate MAPK in *Arabidopsis* suspension cultures (Desikan et al. 1999). Furthermore, Petersen et al. (2000) showed that mutation of the MAPK gene *MPK4* in *Arabidopsis* altered plant defence activation.

H_2O_2 mediates the transcription of specific genes, though the exact mechanism is as yet unknown. Neill et al. (2002) suggested that it could be due to oxidation of cysteine residues of transcription factors. Activation of MAPKs is a common reaction of plant cells in defence-related signal transduction pathways (Neill et al. 2002). Perception of an extracellular signal activates a MAPK, which in turn can facilitate translocation of the signal to the nucleus where it can phosphorylate and activate transcription factors, thereby modulating gene expression (Apel and Hirt 2004; Hirt 1997; Zhou et al. 2004). For example, it has been reported that two tobacco MAPKs, namely salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), are regulated by a common upstream MAPK, which is involved in signalling for PCD (Zhang and Klessig 1998; Zhang et al. 2000). Ren et al. (2006) showed that there was another MAPK, Ntf4, with a similar function to SIPK and WIPK, which, when expressed in transgenic tobacco plants, accelerated the PCD when treated with the elicitin cryptogen from *Phytophthora cryptogea*. This indicates a role in signalling for PCD. Recently, Liu et al. (2007) showed that the combined activation of SIPK, Ntf4 and WIPK induced an HR-like PCD.

Activation of signal transduction could lead to increased ROS accumulation and activation of defence genes coding for PR-proteins, enzymes involved in the generation of phytoalexins, enzymes involved in oxidative stress protection, lignification and other defence responses (Alvarez et al. 1998; Apel and Hirt 2004; Lamb and Dixon 1997). Further evidence for a role of ROS in signalling has come from the fact that addition of low doses of ROS inducers stimulates the induction of detoxification mechanisms, such as SOD and glutathione-*S*-transferase, and activation of other defence mechanisms in neighbouring cells (Levine et al. 1994). Mittler et al. (2004) suggested that NADPH oxidase could be involved in ROS signalling by creating a loop where a small enhancement of ROS production and amplification of the ROS signals occurs in specific cellular locations. Pharmacological and genetic studies (Dat et al. 2003) support the existence of positive amplification loops involving NADPH oxidases in ROS signalling. These loops might be activated by low levels of ROS and result in enhanced production and amplification of the ROS signals. It has been reported that a small GTP-binding protein, Rac, regulates ROS production in rice, most likely through an NADPH oxidase, and induces cell death in rice cells with biochemical and morphological features similar to apoptosis in mammalian cells (Kawasaki et al. 1999). Together, MAPK and calcium-dependent protein kinases seem to play central roles in the regulation of pathogen-responsive NADPH oxidases at the transcriptional and post-transcriptional levels, respectively (Kobayashi et al. 2007).

It has been suggested that the HR is triggered only by balanced production of NO and ROS (Delledonne et al. 1998, 2002)-see also below. More specifically, dismutation of O_2^- to H_2O_2 is required to activate cell death, which depends on synergistic interactions between NO, H_2O_2 and SA (Delledonne et al. 1998; Mur et al. 2006). Scavenging of O_2^- by surplus NO (or vice versa) disturbs the NO/H_2O_2 ratio, resulting in reduced cell death (Mur et al. 2006). Little is known about signalling pathways downstream of NO/H_2O_2. Nevertheless, it has been shown that NO signalling during both PCD and defence responses requires cyclic GMP and cyclic ADP ribose, two molecules that can serve as secondary messengers for NO signalling in mammals (Van Breusegem and Dat 2006).

SA has been shown to be an important signalling molecule involved in defence responses to pathogen attack in many plant–pathogen interactions. Thus, Enyedi et al. (1992) showed that SA levels increased dramatically in tobacco cells surrounding infection sites when infected with *Tobacco mosaic virus*. Torres et al. (2006) suggested that ROS acted synergistically in a signal amplification loop with SA to drive the HR and the establishment of systemic defences. SA accumulation can also down-regulate those ROS-scavenging systems that, in turn, can contribute to increased overall ROS levels following pathogen recognition (Klessig et al. 2000; Shah 2003). In addition to SA, ET and JA are also involved in signalling (Thatcher et al. 2005). The activation of a redox-signalling pathway possessing a MAPK module has also been reported in response to infection by avirulent pathogens in *Arabidopsis* (Suzuki 2002). This signalling network functions independently of the plant hormones ET, SA and JA (Thatcher et al. 2005). Additionally, when some mutants, which develop spontaneous lesions mimicking HR cell death, are placed in a *NahG* background to degrade SA (Shah 2003), lesion formation is suppressed but can be restored by SA treatment (Lorrain et al. 2003). However, this is not the case for all lesion-mimic mutants; some show intensified lesions in plants defective in JA signalling, while others have delayed lesion formation in plants defective in ET signalling (Lorrain et al. 2003). These differences in lesion formation can be due to synergistic or antagonistic effects between SA, JA and ET signalling pathways (Lorrain et al. 2003), but also indicates that mutants displaying the same phenotype could be mutated in widely different genes.

Involvement of ROS in oxidative cross-linking of cell walls

Barriers operating at the cell periphery to prevent invasion represent the first line of defence against pathogens that penetrate plant cells directly (Schulze-Lefert 2004). These barriers can, for example, depend on the nature and thickness of the epicuticular wax layer and cuticle or the composition and physical properties of the cell wall. Alternatively, they may occur by reinforcement of the cell wall, e.g., by deposition of callose-rich papillae and lignin at attempted penetration sites (Heitefuss 1997). ROS production has been associated with the formation of physical defensive barriers (Hückelhoven and Kogel 2003; Lamb and Dixon 1997)-see also Fig. 2b. Association of H_2O_2 with lignification during plant development has been shown in several systems (Olson and Varner 1993; Repka 2002). Thus, H_2O_2 accumulation resulted in lignification in wounded *Zinnia* stem sections (Olson and Varner 1993). Furthermore, Thordal-Christensen et al. (1997) showed that the H_2O_2 production in barley infected with *B. graminis* f.sp. *hordei* led to cell wall cross-linking. Collins et al. (2003) showed that H_2O_2 was associated with vesicles containing cell wall components which were in transit to CWA, suggesting that H_2O_2 may play a role upstream of CWA or that compounds of CWA are oxidatively cross-linked on the way to the site of deposition. These findings have been confirmed by An et al. (2006), who showed that multivesicular bodies, intravacuolar vesicle aggregates and paramural bodies, which might participate in the secretion of building blocks for CWA, are associated with H_2O_2 accumulation. Another conspicuous role of the CWA, besides arresting fungal penetration, is blockage of all plasmodesmata between intact cells and those undergoing the HR, thereby containing the hypersensitive cell death. Also, Iwano et al. (2002) showed that in suspension-cultured rice cells infected with *Acidovorax avenae* (formerly *Pseudomonas avenae*), callose synthesis occurred at the H_2O_2 generation site.

Studies of pearl millet infected with *Sclerospora graminicola* have indicated that cell wall protein cross-linking is induced by enhanced H_2O_2 production at the time of pathogen attack (Kumudini and Shetty 2002). Possibly, hyroxyproline-rich glycoproteins (HRGPs) accumulate and contribute to disease resistance involving cross-linking between HRGP monomers to form a network which provide anchorage for lignification. This might also lead to obstruction of haustorial formation and nutrient shortage, which may be particularly unfavourable for biotrophic pathogens that use specific organs, e.g., haustoria for feeding (Bradley et al. 1992; Shailashree et al. 2004). Likewise, studies on the interaction between wheat and *B. graminis* f.sp. *tritici* showed that H_2O_2 plays important roles in defence, by driving among others the cross-linking to strengthen the cell wall (in effective papillae), and in association with HR (Li et al. 2005).

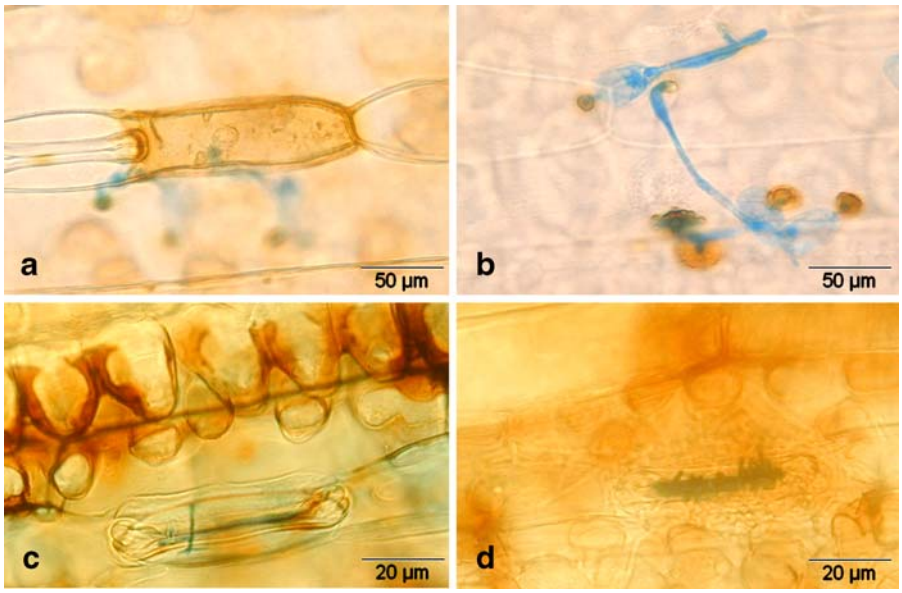

Fig. 2 Accumulation of H_2O_2 as seen by DAB-staining (Thordal-Christensen et al. 1997) in the barley–*B. graminis* f. sp. *hordei* interaction (**a**, **b**) and the wheat–*S. tritici* interaction (**c**, **d**). **a** shows barley isoline P-01 inoculated with isolate c15. A cell is undergoing HR as a response to penetration and is completely stained with DAB. **b** shows barley isoline P-02 inoculated with isolate c15, 2 days after inoculation. Note red–brown staining in the papillae and that the papilla in the cell containing an haustorium is not stained with DAB whereas the other papillae are stained. **c** shows wheat cv. Stakado inoculated with isolate IPO323 of *S. tritici* (incompatible interaction) at 5 days after inoculation. H_2O_2 is accumulating in the apoplast of the substomatal cavity of a stoma penetrated by the pathogen. **d** shows wheat cv. Sevin inoculated with isolate IPO323 of *S. tritici* (compatible interaction) at 15 days after inoculation. H_2O_2 is accumulating throughout the tissue in which fungal sporulation occurs

Involvement of ROS in the hypersensitive response (HR)

The HR is a rapid host response occurring in a host cell, which is infected by a pathogen (Lam et al. 2001; Lam 2004). The cells die shortly after penetration Fig. 2a, often together with some of the surrounding cells (Greenberg 1997; Van Breusegem and Dat 2006). The HR occurs in order to restrict pathogen growth and is highly effective against biotrophic pathogens, since, with the death of host cells, the nutrient supply is removed (Greenberg and Yao 2004; Mellersh et al. 2002; Thordal-Christensen et al. 1997). In addition, toxic substances like ROS and phytoalexins produced in these cells apparently help to kill the pathogen (Lamb and Dixon 1997; Li et al. 2006; Moerschbacher and Reisener 1997). ROS may originate primarily from chloroplasts, mitochondria and peroxisomes (Amirsadeghi et al. 2007; Lam 2004; Op den Camp et al. 2003; Van Breusegem and Dat 2006). The HR is often not effective against necrotrophic pathogens because these usually kill host cells to feed on them (Govrin and Levine 2000; Mayer et al. 2001). Thus, for true necrotrophic pathogens, such as *Botryotinia fuckeliana* (formerly *Botrytis cinerea*), it has been suggested that plant cell death is beneficial for infection, leading to enhanced colonisation (Govrin and Levine 2000; Greenberg and Yao 2004). However, the general nature of this conclusion has been questioned even for *B. fuckeliana* (Unger et al. 2005). In addition, there is a group of pathogens, often considered to be necrotrophic, which are in fact inhibited to some extent by HR, e.g., *Pyrenophora teres* (anamorph *Drechslera teres*; Jørgensen et al. 1998) and *Magnaporthe grisea* (Iwai et al. 2007). Collectively, these findings raise the question whether these pathogens are, in fact, necrotrophic, or should be considered as hemibiotrophic or whether some necrotrophic pathogens may also be inhibited by HR under some circumstances. In our view, the term 'necrotrophic' includes a diverse group of pathogens with quite different modes of pathogenicity.

The HR is a type of active PCD (Greenberg and Yao 2004; Lam 2004; Li et al. 2006; Sasabe et al. 2000; Van Breusegem and Dat 2006), which is often characterised by discrete cellular lesions and preceded

by an oxidative burst (Baker and Orlandi 1995; Dat et al. 2003; Levine et al. 1994; Sasabe et al. 2000). The process of HR may involve several steps including chromatin condensation, DNA cleavage and membrane blebbing, eventually leading to membrane disruption and release of cell contents (Dat et al. 2003; Hoeberichts and Woltering 2003; Lam 2004; Li et al. 2006; Sasabe et al. 2000;). The cell death process thus shares some features with mammalian apoptosis (Greenberg and Yao 2004; Hoeberichts et al. 2003; Hoeberichts and Woltering 2003; Lam 2004). Further similarities include a group of proteins, termed metacaspases, in the genome of *Arabidopsis* with homology to the specific cysteine proteases, termed caspases, which play a key role in execution of mammalian apoptosis (Hoeberichts et al. 2003).

Involvement of ROS in HR has been studied by several different tools, including infiltration of antioxidants (Li et al. 2006) and ROS inhibitors or scavengers (Levine et al. 1994; Li et al. 2006; Sasabe et al. 2000). Furthermore, specific lines of *Arabidopsis* and other plants mutated in their ability to accumulate ROS or express antioxidants (such as SOD, catalase and ascorbate peroxidase) and subsequently activate HR have been studied (Dat et al. 2003; Hoeberichts and Woltering 2003; Jabs et al. 1996; Lorrain et al. 2003; Mateo et al. 2004; Mittler et al. 1999; Montillet et al. 2005; Op den Camp et al. 2003; Torres et al. 2005; Van Breusegem and Dat 2006). Also ROS accumulation has been studied following treatment with pathogen elicitors of HR (Greenberg and Yao 2004; Levine et al. 1994; Montillet et al. 2005; Sasabe et al. 2000). The use of such a diversity of approaches and systems strengthens and substantiates our knowledge on the role of ROS in HR, since broad background information is obtained. However, it also reveals that this process is highly complex, and not yet understood in detail (Van Breusegem and Dat 2006). For example, several studies have shown a correlation between accumulation of ROS (H_2O_2, $^1O_2$, O_2^-), NO and HR (Dat et al. 2003; Floryszak-Wieczorek et al. 2007; Jabs et al. 1996; Levine et al. 1994; Mittler et al. 1999; Montillet et al. 2005; Op den Camp et al. 2003). In this respect, NADPH oxidase has been found to be an important generator of ROS (Lamb and Dixon 1997; Li et al. 2006; Torres et al. 2002). On the other hand, lack of correlation has also been reported in some cases (Dorey et al. 1999; Glazner et al. 1996; Repka 2002; Torres et al. 2005). This discrepancy in results illustrate that, although there is overwhelming evidence that ROS accumulation plays a central role for the HR, we do not yet understand the process or processes in detail and different pathways may operate in different systems or under different conditions. Elucidation of the causal relation between ROS and HR is further complicated by the fact that, for example, plant hormones such as SA, JA, ET and abscisic acid also influence the elicitation and expression of HR (Hoeberichts and Woltering 2003; Torres et al. 2005; Van Breusegem and Dat 2006).

Different models have been proposed to explain how ROS (H_2O_2, O_2^-, $^1O_2^-$) and NO may elicit and regulate HR (e.g., Delledonne et al. 2002; Torres et al. 2005; Van Breusegem and Dat 2006). Thus, when plants are subjected to stress and ROS accumulate at levels insufficient to kill the cell (as opposed to necrosis which is passive, accidental cell death), signalling events lead to PCD represented by HR. Initiation of HR may further lead to activation of other defence responses and systemic acquired resistance (Greenberg 1997; Van Breusegem and Dat 2006).

The exact role and mechanism of ROS in elicitation of the HR remains unclear. For example, it has been suggested from studies of soybean suspension-cultured cells that elicitation of HR requires tightly balanced production of H_2O_2 and NO where NO reacts with H_2O_2 (generated from O_2^- by SOD) and elicits the HR (Delledonne et al. 2001), although it has been questioned whether this is a general phenomenon (Greenberg and Yao 2004). In wheat infected by the hemibiotrophic pathogen *S. tritici*, there was a strong accumulation of H_2O_2 in a resistant cultivar, coinciding with the restriction of pathogen growth and expression of defence genes, but there is no classical HR in the host (Shetty et al. 2003, 2007). Likewise, Sasabe et al. (2000) also found that elicitor treatment of tobacco cell suspension cultures resulted in an oxidative burst, but not in cell death or defence gene activation. This indicates that signalling pathways leading to the oxidative burst, cell death and defence gene activation may branch at an early stage. Also Montillet et al. (2005) found that an HR could be elicited in tobacco by different pathways in light and darkness. There are also reports where elicitors and pathogens have been shown to trigger a strong oxidative burst without causing an HR but activate

other defence mechanisms involved with the oxidative burst (Glazner et al. 1996; Jabs et al. 1997; Repka 2002). For example, Glazner et al. (1996) showed that ROS accumulation in tobacco leaves and cultured cells in response to an incompatible strain of *Pseudomonas syringae* pv. *syringae* was not sufficient to cause HR. Likewise, in parsley suspension-cultured cells inoculated with fungal elicitor, ROS production and activation of defence genes was observed, but no HR, indicating that ROS accumulation could play other roles, such as to act as a direct antimicrobial agent, for induction of defence gene expression and phytoalexin accumulation in the absence of the HR (Jabs et al. 1997).

Clearly, the role of ROS for triggering and executing the HR is complicated and influenced by many factors. Already Levine et al. (1994) suggested that ROS at interaction sites may have different roles in the elicitation or prevention of cell death depending on their concentration, sub-cellular localisation and the duration of their production.

Involvement of ROS in successful pathogenesis

Biotrophic pathogens obtain their nutrition from living host cells (Oliver and Ipcho 2004), and H_2O_2 has been reported as an effective factor in stopping growth of biotrophic pathogens such as *B. graminis* f. sp. *hordei* (Mellersh et al. 2002; Thordal-Christensen et al. 1997; Trujillo et al. 2006). For example, Trujillo et al. (2006) found in the barley–*Blumeria* interaction that H_2O_2 is produced in non-penetrated CWA (see Fig. 2b). However, they also showed that superoxide $\left(O_2^-\right)$ was produced locally at the site of penetration and appeared to enhance infection, thus suggesting that ROS can also be important for the pathogenesis of biotrophic pathogens. Biotrophic pathogens may suppress the host defence responses during infection (Ferreira et al. 2007). For example, the fungal metabolite mannitol, which can suppress ROS-related defence mechanisms by scavenging ROS, was found in apoplastic fluids of *Vicia faba* leaves infected with *Uromyces viciae-fabae* (formerly *Uromyces fabae*; Link et al. 2005; Voegele et al. 2005). On the other hand, HR and ROS such as H_2O_2 have been reported to benefit infection by necrotrophic pathogens, which may even be able to produce ROS themselves or stimulate the host to do so (Govrin and Levine 2000; Von Gönner and Schlösser 1992). For example, Van der Vlugt-Bergmans et al. (1997) studied *B. fuckeliana* infection in *V. faba* and reported that the fungus released H_2O_2 which could destroy host membrane lipids, thereby facilitating penetration. *B. fuckeliana* was also reported to enhance ROS production to aid its tissue colonization by triggering changes in the host (tomato) peroxisomal antioxidant system, leading to a collapse of regulatory mechanisms (Kuźniak and Skłodowska 2005). In vitro studies by Gil-ad and Mayer (1999) showed that *B. fuckeliana* spores could germinate at a concentration of about 180 mM H_2O_2 and that the mycelium could reduce the H_2O_2 present in the culture medium, thus clearly demonstrating that the pathogen could cope with this high H_2O_2 concentration. In contrast, Unger et al. (2005) performed studies in bean leaves and suspension-cultured cells, which indicated that a non-aggressive isolate of *B. fuckeliana* was in fact inhibited by ROS (O_2^- and partly H_2O_2) and HR. On the other hand, an aggressive isolate induced HR-like necrosis and was able to complete its life cycle. The aggressive isolate produced high amounts of a suppressor of O_2^-, i.e., 2-methyl succinate. When this suppressor was added to the non-aggressive isolate, enhanced tissue necrosis occurred. These results demonstrate a situation equivalent to biotrophic pathogens suppressing the host defence responses and indicate that ROS may also inhibit the necrotrophic pathogen *B. fuckeliana* in some cases (c.f. Małolepsza and Urbanek 2002), contrary to previous conclusions regarding this organism.

The so-called hemibiotrophic pathogens are a diverse group of organisms with an initial biotrophic phase where infection is established, followed by a necrotrophic phase where the pathogen completes its life-cycle (Oliver and Ipcho 2004). A correlation between pathogen growth at the late stages of their life-cycle and large quantities of H_2O_2 has also been reported in such host-pathogen systems. Thus, Able (2003) reported that in barley infected by *Rhynchosporium secalis* and *P. teres*, there was a large accumulation of H_2O_2 in compatible interactions in the later stage of infection, and it was concluded that H_2O_2 was necessary for successful infection as for *B. fuckeliana* (see above), however, based only on correlative evidence. Recently, doubts have been raised whether this is a valid conclusion. Shetty et al. (2003) observed a similar correlation in wheat

infected by the hemibiotrophic pathogen *S. tritici*. During the biotrophic phase of the interaction, H_2O_2 accumulation occurred as a defence response only in an incompatible interaction (Fig. 2c). On the other hand, in a compatible interaction, large amounts of H_2O_2 accumulated after extensive tissue colonization just before the symptoms appeared and the pathogen sporulated (Fig. 2d). However, Shetty et al. (2007) found that, even though sporulation of *S. tritici* in wheat coincided with a massive accumulation of H_2O_2, removal of this H_2O_2 by infiltration of catalase resulted in increased pathogen growth, indicating both that it can survive and tolerate the presence of H_2O_2, but also that this H_2O_2 inhibits the pathogen.

The ability to colonise and proliferate in an environment with high concentrations of ROS shows that the pathogens have efficient systems enabling them to protect themselves against the harsh environment in the host. Thus, for the necrotrophic pathogen *B. fuckeliana*, Van der Vlugt-Bergmans et al. (1997) found that it could protect itself by expressing genes encoding catalase which could scavenge H_2O_2. In accordance with this, Goodwin et al. (2001) cloned a catalase gene from the hemibiotrophic pathogen *Colletotrichum gloesporioides* f.sp. *malvae* (pathogen of *Malva pusilla*). Catalase genes have also been reported from *S. tritici* (Levy et al. 1992), but their expression was not studied. However, it can be predicted that catalases will be activated during the necrotrophic phase of *S. tritici* growth to help protect the pathogen from the deleterious effect of H_2O_2. In agreement with this, Shetty et al. (2003) found high levels of catalase activity in a susceptible host during pycnidial formation, but were unable to determine whether this was of host or pathogen origin.

Other types of hemibiotrophic pathogens might benefit from ROS accumulation at some stage. Thus, Kumar et al. (2001) reported that barley with *mlo* resistance against *B. graminis* f.sp. *hordei* was very susceptible to *Bipolaris sorokiniana*. Toxins from *B. sorokiniana* killed the host cells, leading to massive H_2O_2 accumulation, and it was hypothesised that the *mlo*-resistant plants were very susceptible because cell death occurred more easily compared to other barley genotypes. After cell death, the pathogen could grow unhindered in the dead tissue and was not inhibited by H_2O_2 accumulation (cf. Fig. 1). However, in the initial stages of penetration, it was concluded that *B. sorokiniana* was inhibited by H_2O_2 accumulation just beneath appressoria from which penetration was attempted, i.e., before cells died.

Most conclusions regarding the role of ROS in successful pathogenesis are based solely on correlative data and come from rather few pathosystems. *B. fuckeliana* is most often used as a representative necrotrophic pathogen. The influence of ROS on this pathogen is fairly well studied even though conflicting results have been reported, but generalisations to other pathogens should be made with caution. Thus, as pointed out by Shetty et al. (2007), even if there is a correlation between ROS accumulation and pathogen colonisation, the fact that the pathogen can tolerate the presence of large amounts of ROS does not necessarily mean that it benefits from ROS. Furthermore, there is disagreement as to which categories different pathogens belong (see, e.g., Oliver and Ipcho 2004). For example, *R. secalis* was reported to be necrotrophic (Able 2003), whereas previous research has shown this pathogen to have a long symptomless phase (Jørgensen et al. 1993), suggesting that it should be considered as hemibiotrophic. Therefore, caution should also be taken when concluding about a definite role of ROS for specific types or even species of pathogens before thorough studies have been conducted.

Discussion

Although our understanding of the oxidative burst in plant–pathogen interactions has advanced considerably since the first reports, there are still several unanswered questions. Thus, Fig. 1 shows an overview of our current knowledge of the different roles of ROS in host–pathogen interactions, but also indicates some of the areas where there are unanswered questions and gaps in our knowledge.

The rapid production of ROS in the apoplast in response to pathogens has been proposed to orchestrate the establishment of different defensive barriers against pathogens (Torres et al. 2006). Our understanding of sources and roles of ROS has been greatly enhanced by the identification of defence-associated mutants in the model plant *A. thaliana* (Lorrain et al. 2003). These mutants have not only allowed the identification of important signalling intermediates but also allowed the dissection of ROS-mediated

signalling pathways. However, the influence of other factors such as environment, plant hormones and activation of different signalling pathways (Op den Camp et al. 2003; Montillet et al. 2005; Sasabe et al. 2000; Torres et al. 2005) plays an important role for the accumulation of ROS, and this needs to be taken into consideration and studied in detail. Likewise, external factors such as different types of pathogens and elicitors may vary in their ability to trigger ROS production (cf. Fig. 1), and are therefore possible reasons for conflicting results. This emphasises the need for studies of several different host–pathogen systems in order to clarify if and when different pathways are activated in different situations. It is therefore essential to study several different hosts infected by taxonomically different pathogens which represent different life-style strategies before making general conclusions and thus avoid over-simplification. There are profound differences between monocots and dicots as well as in the biology of biotrophic, hemibiotrophic and necrotrophic pathogens. Caution should therefore be exercised before stating that processes occur in a similar way in totally different systems.

It is also important to adopt different approaches to increase the robustness of conclusions as illustrated by the example of involvement of ROS in HR. Besides a genetic approach, using mutants, gene silencing, gene knock-outs and/or over-expression, careful physiological and biochemical characterisation of different host–pathogen interactions and defence responses activated should be carried out followed by studies of the role of proteins encoded by ROS genes in the different systems. This approach provides insight into their precise function in defence, cell death, and/or pathogen development, through determination of their sub-cellular localisation and biochemical function. In particular, in relation to the evaluation of the role of ROS in successful pathogenesis, it is important to try to inhibit the cell death machinery selectively and simultaneously to monitor other defence and pathogenesis-related events. Using this approach, it should be possible to determine whether cell death can be uncoupled from other defence responses and if so, the specific contribution to resistance or susceptibility in the interaction in question. Of particular interest in this context is to determine which role ROS plays in HR/necrosis against necrotrophic pathogens (cf. Fig. 1). Thus, do these pathogens all benefit from ROS accumulation or are some of them actually inhibited to some extent by ROS or other substances in the dying cells? Another important question regarding necrotrophic pathogens relates to potential toxins produced (cf. Fig. 1). Thus, will those toxins which kill host cells always cause the release of ROS, which in turn causes an HR (cf. *B. sorokiniana*)? It is also important to study the interplay between ROS and SA/NO, in order to gain further insights into the regulation of resistance, as these are important defence response regulators that interact with ROS signalling in response to pathogens (Mur et al. 2006). Thus, ROS may be part of many signalling pathways and provide a crucial link in the cross-talk to different responses (Apel and Hirt 2004).

The flux of information between different cell compartments needs to be elucidated to further understand the regulatory capabilities of ROS. Previously, genetic engineering for improved disease resistance has mainly targeted genes involved in the recognition of the pathogen or in the over-expression of defence molecules like phytoalexins (Jalali et al. 2006). An interesting alternative approach would be to target key molecules like ROS that act at points of convergence of different signalling pathways. Engineering plants with such genes using a pathogen-inducible promoter would enable expression of different downstream genes simultaneously in the host, ensuring that plants develop an array of effective responses, which will ultimately secure a sustainable and durable resistance against a range of plant pathogens.

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